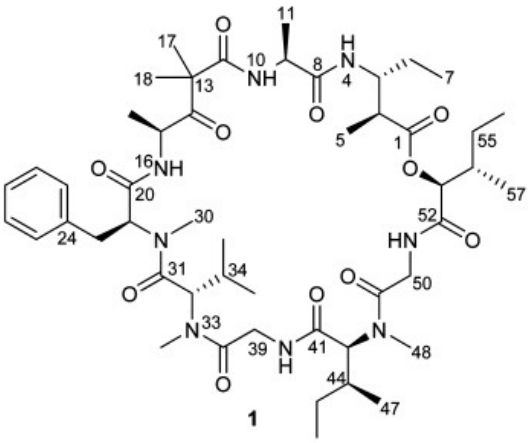
Synthesis of N-protected γ-amino-β-keto esters and applications of Isothermal Calorimetry.

Gamma- amino β-keto acids are highly versatile non-natural amino acids present in several biologically active peptides.[1,2] Some of the naturally occurring peptides containing b-keto-g-amino acids are shown in Figure 1. Furhter, γ- Amino β-keto esters have been widely used as intermediates for synthesis of many biologically relevant molecules such as statins[3a,b], ketomethylene dipeptide isosteres[3c], β-lactams[3d], tri carbonyl compounds[3e], rhodopeptins[3f] , substituted pyridines[3g], flouroscent amino acid tags[3h,i], cholecystokinin(CCK) receptor antagonists[3j], γ-amino α,β-unsaturated esters[3k], and α-azo-β carbonyl compounds[3l]. Several synthesis routes have been reported for preparation of γ-amino β-keto esters[4]. The most commonly reported synthesis route involves claisen ester type condensation of activated carboxylic acid followed by nucleophilic substitution either with lithium enolates of alkyl acetates[3b,5a-c] or magnesium enolates of alkyl malonates or mono alkyl malonates in the presence of magnesium chloride.[3a,l]



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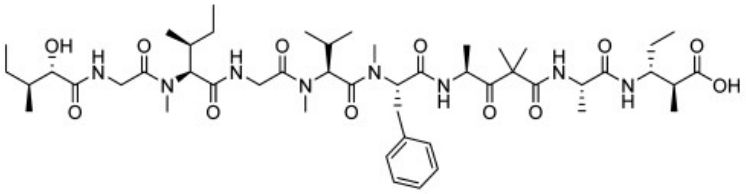

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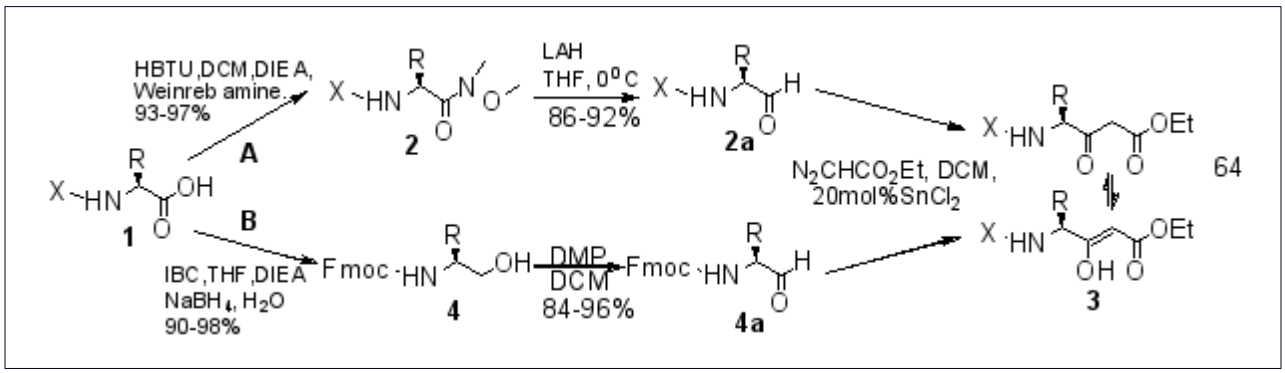
Figure 1. Molecular structures of cyclic DMMC (**1**) and its linear hydrolysis product (**2**).

N',N'-Carbonyl diiimidazole(CDI) is normally used for carbonyl activation[6]. The other carbonyl activation groups are pentaflourophenyl esters[5a], N-carboxyandrides(NCA)[7a,b] and mixed anhydrides of N-protected amino acids[5b]. Similarly C- acylation of protected amino acids with Meldrum's acid via mixed anhydride method followed by the hydrolysis leading to β-keto esters have also bee reported[8]. Different Success rates depend on various factors such as functional-group compatibility, multistep procedures for nucleophilic preparation , poor yields , harsh Reaction conditions and duration of reaction. Thus , there is a need for the development of new and efficient methods for synthesis of γ- Amino β-keto esters.

Formation of ketones from reactions between diazomethane and aldehydes is a very classic organic reaction.[9] The versatility of metal mediated diazo coupling reactions is well reported [10]. Also participation of diazoacetate in aldol[11] and Mannich[12] type reaction in presence of lewis acids catalysts are reported and documented. Recently, Holmquist and Roskamp reported lewis acid mediated synthesis of rearranged β-keto esters from aliphatic and aromatic aldehyded with ethyl-diazoacetates.[13]

Aim of this was synthesis of γ- Amino β-keto acids containing peptides and their future biological activity. The planning was based on the reaction route that γ- Amino β-keto acids could be obtained from the reaction between N-Protected amino aldehydes and ethyl diazoacetate as shown scheme 1.



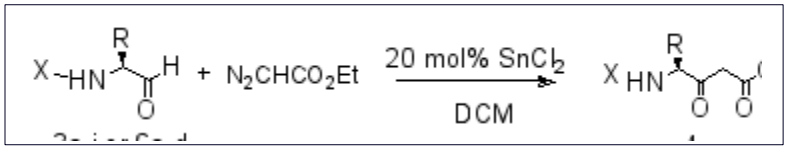
Scheme 1:

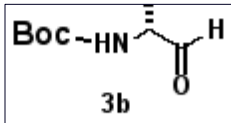
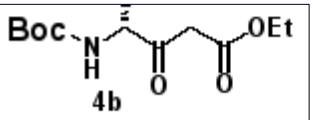
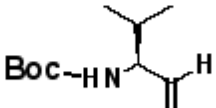
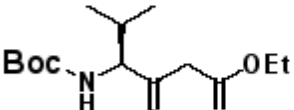
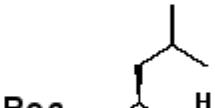
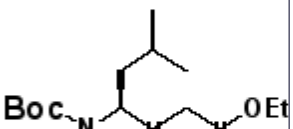
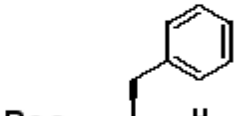
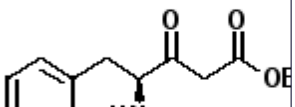
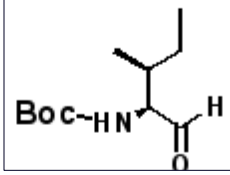
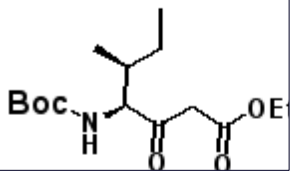
In this report, a facile and efficient synthesis of γ- Amino β-keto esters using N-protected amino aldehydes and ethyl diazoacetate in presence of a catalytic amount of anhydrous tin chloride(+2) at ambient temperature.

Results and Discussions.

The feasibility of this method was explored with Boc-Protected amino aldehydes , Scheme1A shows the synthesis of amino aldehydes starting from Weinreb amide2. The protected amino acid 1 was converted to 2 following the reported procedure[14]. The column purified Weinreb amides were subjected to LAH reduction to give corresponding aldehydes.

Table 1. List of N-protected γ-amino β-keto esters synthesized from the N-protected aldehydes and ethyl diazoacetate in the presence of 20 mol% tin(II) chloride.



Entry	substrate	product	yield(%)
	 3b	 4b	
	 3c	 4c	
	 3d	 4d	
	 3e	 4e	
			

The amino aldehydes thus produced were directly used for the next step without purification. In a typical reaction procedure 3a was dissolved in DCM and pre-activated by adding 20 mol% of anhydrous $SnCl_2$ at room temperature. To this ethyl diazoacetate was added slowly and nitrogen gas was found to be evolving for next 30 mins indicating the complete consumption of aldehyde. It was found out that about 20% of catalyst is optimum for the reaction .

Different lewis acid catalysts such as Zinc chloride , Boron triflouride and titanium tetrachloride were also reported in a similar type of reaction[13,15] ; however this synthesis was restricted to $SnCl_2$ because Zinc lewis acid catalysts have also been used for Mukaiyama-aldol type reactions[11c] with aldehydes and diazoacetates. In addition, Boron fluorides have been reported with lower yields compared to tin lewis acids[16].

Further $SnCl_2$ mediated reactions were found to be working fine in normal conditions and thus the reactions were performed in open flasks.

In a control reaction however when no catalyst was used for coupling reaction , no reaction occurred and the starting aldehyde was isolated.

This method was further extended to other Boc-Protected amino aldehydes. The reactions were instantaneous and all Boc-protected β-keto esters were isolated in good yields(76-84%) and purity after simple aqueous work-up and are given in Table 1. The yields come out to be better than nucleophilic substitution.

The ^{1}H and ^{13}C NMR of the N-protected γ- Amino β-keto esters were recorded in CDCL3. A doublet of doublet for α-active methylene protons in AB coupling pattern at δ 3.5ppm along with two distinct and common peaks at around δ 5.5 and δ 12ppm for enol protons . The ratio of keto/enol form was found to be > 10:1. Notably, we did not observe any change in the ratio of keto/enol tautomers even with a range of temperatures from 233K-303K.

Experimental Section

All amino acids , ethyl diazoacetate, LAH, DiPEA, stannous chloride were purchased from Aldrich. THF,DCM and DMF were purchased from Merck. Isobutyl Chloroformate, Sodium borohydride , HBTU, HOBT, di-tert-butyl dicarbonate were obtained from spectrochem and used without further purifications.

THF and DiPEA were dried over sodium and distilled immediately prior to use. Column Chromatography was performed on Merck silica gel (120-200 mesh). The ^{1}H spectra were recorded on Brucker 500MHz (or 125 MHz for ^{13}C) . and Jeol 400 MHz (or 100 MHz for ^{13}C) spectrometers using residual solven signals as an internal reference ($CDCl_3$,δH,7.24ppm,δC 77.0ppm). The chemical shifts (δ) are reported in ppm and coupling constants(J) in Hz. High-resolution mass spectra were obtained from HRMS-ESI(waters) , LCMS/MS(Waters) and MALDI TOF/TOF(Applied Biosciences)

General Procedures for the synthesis of N-Protected γ- Amino β-keto esters.

The N-Protected amino aldehyde (2.0mmol) was dissolved in 15mL of DCM at room temperature (293-298K) and then 0.0756g (20mol%) of stannous chloride was added followed by 0.239g(2.1mmol) of ethyl diazoacetate. Immediate gas evolution was observed. The reaction mixture was stirred and observed with TLC. After completion of the reaction , it was quenched with 10 mL of 0.5N HCL and the reaction mixture was extracted with DCM (30mL*3) . The combined organic layer was washed with 20mL of brine , dried over anhydrous sodium sulfate and concentrated under reduced pressure to get a greenish oily crude product which was purified on silica gel column chromatography.

(S)-ethyl 4-(tert-butoxycarbonylamino)-3-oxopentanoate (4a): The representative procedure was employed for the coupling of **3a** with ethyl diazoacetate. The procedure gave the title compound **4a** as a Colorless liquid (0.398g, 76%) after purification by column chromatography with 15% ethyl acetate/ pet-ether (60-80 ºC) as the eluent. ^{1}H NMR (500MHz, $CDCl_3$) δ 12.138 (s, 1H enolic 3.5%), 5.173 (b, s, 1H, NH), 4.434-4.378 (m, 1H, CH), 4.244-4.202(q, *J*=7, 2H, OCH_2), 3.625-3.541 (dd, J=14.5, J=10.5 2H, CH_2, AB coupling), 1.467 (s, 9H, $C(CH_3)_2$, Boc), 1.389-1.367 (d, J=6.5, 3H, CH_3), 1.317-1.289 (t, J=7, 3H, CH_3); ^{13}C NMR (500MHz, $CDCl_3$) δ 202.50, 166.96, 155.19, 80.14, 61.57, 55.42, 45.91, 28.32, 17.11, 14.11; HR-MS m/z calcd for [M+Na$^+$] 282.1317, obsrvd. 282.1317; $[\alpha]_D^{25}$= -35.69 (c=1, MeOH, Lit [7b] $[\alpha]_D^{25}$= -24) .

(*R*)-ethyl 4-(*tert*-butoxycarbonylamino)-3-oxopentaanoate (4b) : Colorless liquid (0.405 g, 78%) ; **^{1}H NMR** (400MHz, $CDCl_3$) *δ* 12.0917 (s, 1H enolic 7.5%), 5.11386-5.1248(d, *J*=5.5 Hz, 1H, NH), 4.4852-4.3383 (m, 1H, CH), 4.2050 -4.1512(q, *J*=7.3 Hz, 2H, CH_2), 3.5853-3.4868 (dd, *J*=14.5 Hz, J=10.5 Hz, 2H, CH_2, AB coupling), 1.4226 (s, 9H, $C(CH_3)_3$, Boc-), 1.3459-1.3275 (d, *J*=7.3 Hz, 3H, CH_3), 1.2748-1.2393 (t, *J*=7.3 Hz, 3H, CH_3); **^{13}C NMR** (100MHz, $CDCl_3$) *δ* 202.5561, 167.0207, 155.2455, 80.1988, 61.6349, 55.4851, 45.9696, 28.3783, 17.1752, 14.1623 ; **MALDI TOF/TOF**- m/z Calcd. for $C_{12}H_{21}NO_5$ [M+Na]$^+$ 282.1317, obsrvd 282.1317; $[\alpha]_D^{25}$ = +35.16 (c = 1, MeOH).

(*S*)-ethyl 4-(*tert*-butoxycarbonylamino)-5-methyl-3-oxohexanoate (4c) : Colorless liquid (0.48g, 84%); **^{1}H NMR** (500MHz, $CDCl_3$) *δ* 12.118 (s, 1H enolic form 6.5%), 5.067 (s, b, 1H, NH), 4.351-4.321 (m, 1H, CH), 4.224- 4.181 (q, *J*= 7 Hz, 2H, -OCH_2), 3.572-3.505 (dd, *J*=15.5 Hz, *J*=3 Hz, 2H, CH_2, AB coupling), 2.270-2.239 (m, 1H, $CH(CH_3)_2$), 1.449 (s, 9H, $C(CH_3)_3$, Boc-), 1.294-1.266 (t, *J*=7 Hz, 3H, CH_3), 1.029-0.822 (m, 6H, $C(CH_3)_2$); **^{13}C NMR** (125MHz, $CDCl_3$) *δ* 202.23, 166.75, 155.86, 80.03, 64.38, 61.55, 47.14, 29.56, 28.31, 19.84, 16.67, 14.10; **HR-MS** m/z Calcd. for $C_{14}H_{25}NO_5$ [M+Na]$^+$ 310.1630, obsrvd. 310.1620 ; $[\alpha]_D^{25}$ = - 32.64 (c = 1, MeOH), Lit .

(*S*)-ethyl 4-(*tert*-butoxycarbonylamino)-6-methyl-3-oxoheptanoate (4d) : Light yellowish liquid (0.476g, 79%); **^{1}H NMR** (500MHz, $CDCl_3$) *δ* 12.107 (s, 1H, enolic 5.5 %), 4.966- 4.947 (d, *J*=9.5 Hz, 1H, NH), 4.402-4.361 (m, 1H, CH), 4.246-4.207 (q, *J*=7 Hz, 2H, -OCH_2), 3.633-3.532 (dd, *J*=16.0 Hz, *J*=18.5Hz, 2H, CH_2, AB coupling), 1.740-1.677 (m, 3H, CH_2, CH), 1.469 (s, 9H, $C(CH_3)_3$, Boc-), 1.321-1.293 (t, *J*=7 Hz, 3H, CH3), 0.979 (b, s, 6H, $C(CH_3)_2$); **^{13}C NMR** (125MHz, $CDCl_3$) *δ* 203.02, 167.07, 155.55, 80.15, 61.51, 58.19, 46.35, 39.90, 28.31, 24.83, 23.28, 21.59, 14.12; **HR-MS** m/z Calcd. for $C_{15}H_{27}NO_5$ [M+Na]$^+$ 324.1786 obsrvd. 324.1784 ; $[\alpha]_D^{25}$ = - 53.70 (c = 1, MeOH, lit.[3a] $[\alpha]_D^{25}$= -51.3).

(*S*)-ethyl 4-(*tert*-butoxycarbonylamino)-3- oxo-5-phenylpentanoate (4e) : White crystal (0.521 g, 78%); Melting Point = 61.4^0 C; **^{1}H NMR** (400MHz, $CDCl_3$) *δ* 12.1616 (s, 1H, enolic 17%), 7.2474 –7.1501 (m, 5H, C_6H_5), 5.0366-5.0183 (d, *J*= 7.3 Hz, 1H, NH), 4.5796-4.5257 (q, *J* =6.4 Hz, 1H, CH), 4.1821-4.1283(q, *J* = 7.2 Hz, 2H, -OCH2), 3.5143-3.4054 (dd, *J*=16 Hz, *J*=11.4 Hz, 2H, CH2, AB coupling), 3.1592-2.9518 (m, 2H, CH_2Ph), 1.3802(s, 9H,

$C(CH_3)_3$), 1.2634-1.2279 (t, *J*=7.2 Hz, 3H, CH_3); **^{13}C NMR** (100MHz, $CDCl_3$) δ 201.9697, 166.8634, 155.1835, 136.0477, 129.2400, 128.6774, 127.0089, 80.2132, 61.4586, 60.4384, 46.8612, 36.8976, 28.2020, 14.0241; **HR-MS** m/z Calcd. for $C_{18}H_{25}NO_5$ $[M+Na]^+$ 358.1630, obsrvd 358.1633 ; $[\alpha]_D^{25}$ = - 54.5 (c = 0.6, MeOH, lit.[3a] $[\alpha]_D^{25}$= -56.3).

(*4S, 5R*)-ethyl 4-((*tert*-butoxycarbonyl)amino)-5-methyl-3-oxoheptanoate (4f) : Colorless liquid (0.453g, 76%); **^{1}H NMR** (400MHz, $CDCl_3$) δ 12.0963 (s, 1H, enolic 7.5%), 5.0335-5.0149(d, *J*=8.24 Hz,1H, NH), 4.3161-4.2876 (m, 1H, CH), 4.1993-4.1374 (q, *J*=6.88 Hz, 2H, -OCH2), 3.5131(s, 2H, αCH_2), 1.9723-1.9083 (m, 1H, CH), 1.6328-1.5782 (m, 2H, CH_2),1.4169 (s, 9H, $C(CH_3)_3$, Boc-), 1.2703-1.2348 (t, *J*=7.2 Hz, 3H,CH_3), 0.9793-0.9541(dd, *J*=3.64 Hz, *J*=3.24 Hz, 3H, CH_3), 0.8923-0.8556 (t, *J*=6.9 Hz, 3H, CH_3); **^{13}C NMR** (100MHz, $CDCl_3$) δ 202.3871, 166.7278, 155.7440, 79.9917, 64,2692, 61.4756, 47.2881, 36.2852, 28.2571, 24.0047, 16.0242, 14.0506, 11.6097; **MALDI TOF/TOF**- m/z Calcd. for $C_{15}H_{27}NO_5$ $[M+Na]^+$ 324.1787, obsrvd. 324.1709; $[\alpha]_D^{25}$= - 25.08 (c = 1, MeOH, lit.[7b] $[\alpha]_D^{25}$ = -23).

Chapter 2:

Isothermal titration calorimetry and its uses in bio molecular reactions.

Isothermal titration calorimetry(ITC):- It is one of the best analysing standard for measuring biomolecular interactions. It simultaneously determines all binding parameters like change is gibbs free energy ,Binding constant, enthalpy change, and change is entropy. Thus it indicates spontaneity , disorderness , rate of forward and backward reaction, equilibrium if attained, and its exothermic or endothermic behaviour.

The relation used by this method is :-

$\Delta G = -RT\ln K_a = \Delta H - T\Delta S$

ΔG is change is its free energy.

R is the gas constant.

T is the absolute temperature.

Ka is the forward binding constant.

ΔH is the change in enthalpy.

ΔS is the change in entropy.

When substances bind, heat is either generated or absorbed .Since the reactions are done in constant pressure, Heat change can be taken as enthalpy change which is better to use *s*ince it is a state function.ITC measures directly the heat released or absorbed during binding or dissociation of bio molecular entities .The accurate measurement of the enthalpy change allows us the direct and accurate determination of other thermodynamic parameters of binding/dissociation like binding constant(Kb),reaction stoichiometry(n),entropy change(ΔS) and ultimately, ΔG. Since ITC goes beyond finding binding capacity, it has become the method of choice currently super ceding other instruments such as DSC(Differential scanning calorimetry).

Applications of Isothermal Titration calorimetry:-

1. Interaction between two small molecules .like

Protein-small molecule

Protein-Protein

Target-Drug

Enzyme-inhibitor

Antibody-Antigen

Protein-DNA

Protein-Lipid

2. Assessment of the effect of molecular structures changes on binding mechanisms.

Benefits of ITC:-

1. True affinity data using heat measurement gives a unique insight into biological recognition processes.
2. Directly measure sub-millimolar to nanomolar binding constants.
3. No labelling or immobilization required, also there is no buffer restriction.
4. It also gives number of binding sites, information about multiple binding sites.

Thermodynamic parameters, Meaning and definitions.

A. Enthalpy(H).

Mathematically, Enthalpy is defined as $H=U+PV$.

Thus at constant pressure ,$dH = dU+PdV$.

We know that $-PdV$ is the work done and also from the first law of thermodynamics, $U=Q+W$. ,Thus $dQ=dU-W$; $dQ=dU+PdV$.

It can be seen that dH is nothing but dQ at constant pressure, Also, Enthalpy is a state function which is related to temperature as $dH=nCpdT$,Where dH is the change is enthalpy, n is the number of moles ,Cp is the molar heat capacity at constant pressure and dT is the change in temperature.

Theoretically, Enthalpy is a measure of the total energy of a thermodynamic system. It includes the internal energy,and the amount of energy required to make room for the system by displacing some volume. Enthalpy change(Hf-Hi) is more useful than knowing the absolute value of enthalpy as it gives an indication about whether the system is loosing energy to sorroundings (Exothermic reaction and dH is negative) or

the system is taking the heat from surrounding (Endothermic reaction and dH is positive).

Knowing the signs of dH is very important in Biochemical reactions as if the reaction attains or tends to attain equilibrium then using LeChatlier principles we can attain maximum optimized amount of products.

Entropy(S);-

In classical Thermodynamics, Concept of entropy is defined by the second law of thermodynamics, which states that the entropy of an isolated system always increases or remains constant. Entropy is an indication of randomness of a system.

"Any method involving the notion of entropy, the very existence of which depends on the second law of thermodynamics, will doubtless seem to be many far-fetched, and may repel beginners as obscure and difficult of comprehension."-Williard Gibbs, Graphical methods in Thermodynamics of Fluids.(1873).

Entropy mathematically is Qrev/T where Qrev is the heat change in a reversible process. Entropy is a state function With SI unit J/KInitially, it was contended through the second law of thermodynamics that only such processes with increase in entropy will be spontaneous. But it was found out to be not true always. Both the enthalpy change and the entropy change were the factors behind the spontaneity of a process. This thought finally culminated into a new State function ,Gibbs Free Energy.

Gibbs Free Energy:-

Some Reactions are Spontaneous because they give up energy in the form of heat(ΔH =-ve) and some reactions are spontaneous because lead to increase in the disorder of a system (ΔS=+ve).

The ambiguity of both the parameters in finding the spontaneity is removed by introducing Gibbs Free Energy,(G).

The Gibbs free energy at any moment is defined as the enthalpy of the system minus the product of the temperature (in K) times the entropy of the system.

$G=H-TS$

This is a state function ,since it depends only on state functions.Thus,

$\Delta G = \Delta H - \Delta(TS)$.

At constant temperature,

$\Delta G = \Delta H - T\Delta S$.

This change can be studied under any set of conditions and if

The data are collected under standard –state conditions,the result is the standard –state free energy of activation.(ΔG^0).

Standard conditions:-A process for which the temperature is 273 K and the pressure is 100Kpa(approximately 1 Atm).

Thus at STP(Standard temperature and Pressure)

$\Delta G^0 = \Delta H^0 - T\Delta S^0$

The beauty of this equation is its ability to determine the relative contribution of both the driving forces, Enthalpy change and the Entropy Change to the spontaneity of a reaction.

Favourable or Spontaneous reactions are the one with $\Delta G^0 < 0$ while an Unfavourable or non-Spontaneous reactions are the one with $\Delta G^0 > 0$.

Spontaneous reactions are termed as exergonic and Non-Spontaneous reactions are termed as endergonic reactions.

As we can see from the equation for Gibbs free energy change, for a reaction to be spontaneous , ΔH should be negative(exothermic) and ΔS should be Positive. Also if entropy decreases by a small value high negative value of enthalpy change will compensate the whole reaction to be spontaneous, similarly an endothermic reaction can also be spontaneous if entropy change associated with the process is too positive.

The SI Unit of G,H,TS are Joule.

Relation between the standard Gibbs free energy change and Equilibrium.

Reaction Quotient:

It is defined as the ratio of product of molar concentration of products to the product of molar concentration of reactants.

At equilibrium Reaction Quotient(Qp) is Equal to Equilbrium Constant(Kp).

When Qp=1, $\Delta G = \Delta G^{0.}$

When Qp=Kp. $\Delta G = 0$.

The relationship between the free energy of reaction at any time of the reaction (ΔG) and the standard –state free energy of reaction (ΔG^0) is described by the following equation:-

$\Delta G = \Delta G^0 + RT\ln Q$

R is the ideal gas constant , T is the temperature in Kelvin ,ln represents the logarithm to the base e, and Q is the reaction quotient at the time.

Since, the Driving force (ΔG) is zero at equilibrium (since the rate of both the forward and the backward reactions are same).

$0 = \Delta G^0 + RT\ln K$

$\Delta G^0 = -RT\ln K$

The key to understand the relationship between K and ΔG^0 is the fact that the smaller the value of ΔG^0, the closer the standard state is to equilibrium. The larger the value of ΔG^0 , the further the reaction has to go to reach equilibrium.

Thus, ΔG^0 gives us an idea about both the spontaneity of a reaction and how close the reaction is to attain the equilibrium.

Binding Constant/Binding Affinity.(Kb).

The Starting point of most thermodynamic studies is the experimental determination of the equilibrium association or binding constant (Kb)

Figure 1.1 A schematic diagram to illustrate two basic phenomena that are often the focus of biophysical studies: biopolymer thermal stability and biopolymer–ligand interactions. All these equilibria can be studied using a combination of ITC and DSC. The blue spheres in the DNA complex represent Zn^{2+} ions

What is Binding Constant?

The binding constant is a special case of the equilibrium constant K which is associated with the binding and unbinding reaction of receptor (R) and ligand (L) molecules which is formalized as:-

$$R + L \rightleftharpoons RL$$

This reaction is characterized by the on-rate kon and koff which are the rate constant for the forward reaction and backward reaction respectively.

K_a=[RL]/[R][L]=kon/koff

Where [R],[L] and [RL] represent the concentration of unbound free receptors , the concentration of unbound free ligands and concentration of receptor-ligand complexes.

Sometimes dissociation is more prominent than association. In that case Kd is the term which is considered more compared to Ka.

Kd=1/Ka

For a binding interaction the equilibrium constant can be determined accurately in a number of ways, most of which rely upon measuring the concentrations of free and bound ligand.

The stability of a bounded ligand can be studied based on the value of Kb. Higher the value of Kb Higher will be amount of bonded macromolecule/host we intend to study.

C_p (molar heat capacity at constant pressure).

Both internal energy and enthalpy are state functions.

From the first law of thermodynamics and definition of enthalpy we know that

U=Q+W

And H=U+PV

Where the symbols represent the usual notations.

In the case of reversible processes,w=-PdV

For Processes under constant pressure,

dH=dU+PdV

thus, dH=dQ

$dU=nC_vdT$ (for all the processes)

$dQ=nC_pdT$(for isobaric processes)

thus $dH=nC_pdT$ (for all processes,since enthalpy is a state function).

C_v= molar heat capacity at constant volume.

dT=Change in temperature.

n= number of moles of gaseous entities.

Thus we can deduce that $C_p=dH/dT$ for 1 mole of gas.

Calculation of Heat capacity change.

A single ITC assay can provide information regarding the binding constant , the gibbs free energy of binding , the binding enthalpy, the binding entropy and the stoichiometry of the binding reaction.

In addition to this information , the change in heat capacity upon binding and the change in the ionisation state upon binding can be obtained by repeating the experiment with varying temperature .

Using the derivation before, one can determine the value of Cp by measuring the enthalpy change of a binding reaction at different temperatures. Plotting ΔH versus temperature would give a straight line with slope as ΔC_p.

The heat capacity of binding reflects the burial of polar and non-polar surfaces as a consequence of the binding reaction [17] .

ΔC_p is usually determined by measuring the enthalpy of binding from 298K to 318K at 5 centigrade intervals under identical buffer and pH conditions.

Observed Enthalpies arise largely as a result of changes in interatomic interactions , the most important in biological systems being Hydrogen Bond. The magnitude of the interaction enthalpy is dependent on Bond length and bond angles. However the sign indicates whether there is a net favourable(-ve; exothermic) or unfavourable(+ve; endothermic) redistribution of the hydrogen bond network between the reacting species including solvent. Hydrophobic interactions are related to the relative degrees of disorder in the free and bound systems and therefore these interactions are reflected in the entropy change. The release of water molecules from a wet surface to the bulk solvent is a common source of favourable entropy. This, coupled with the inability of non-polar groups to hydrogen bond with the surrounding water molecules, is the main reason for the strong energetic influence of hydrophobicity in biology.

The thermodynamic signature of this force is typically characterized by a small enthalpy, either positive or negative, and a favourable entropy. A large negative Cp is thought to arise from the accommodation of non-polar groups by water and is therefore another useful indicator of hydrophobic interactions.

The heat capacity change of binding processes between a protein and a ligand are usually negative and less than 1Kcal/K.mole in absolute value. However, the binding of two macromolecules can be associated with a higher heat capacity change, which is indicative of a large burial of solvent-accessible surface area upon binding or the structuring of some regions of the macromolecule[18].

The working of an Isothermal Calorimeter.

In a typical ITC experiment, a solution of a one biomolecule(ligand such as a drug, protein,DNA molecule,etc.) is titrated into a solution of its binding partner. The heat changed during the interaction is monitored over time Figure 2 .Each peak represents a heat change associated with the injection of a small volume of sample into the ITC reaction cell. As successive amounts of the ligand are titrated into the ITC cell , the quantity of heat absorbed or released is in the direct proportion to the amount of binding. As the system reaches saturation, the heat signal diminishes until only heat of dilution is observed. A binding curve is then obtained from a plot of the heats from each injection against the ratio of ligand and binding partner in the cell (figure 2a). The binding curve is analyzed with the appropriate binding model to determine Kb, n and ΔH.

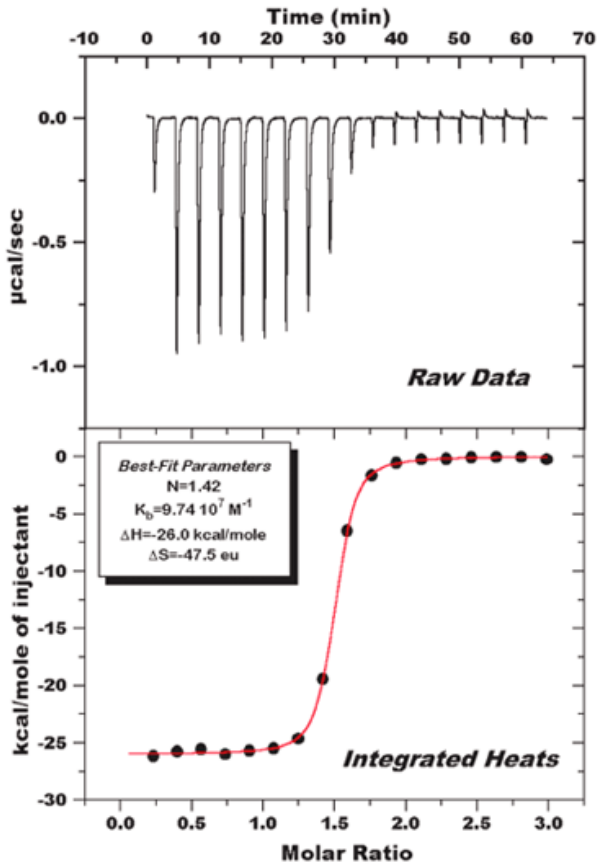
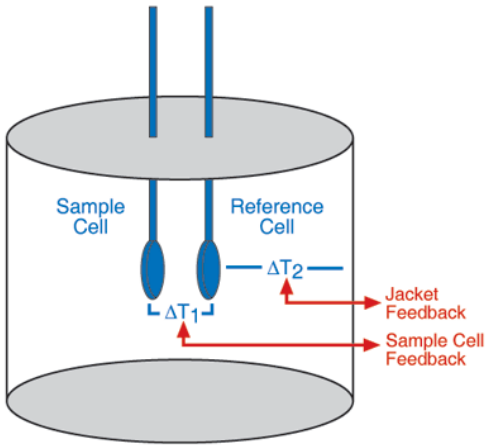


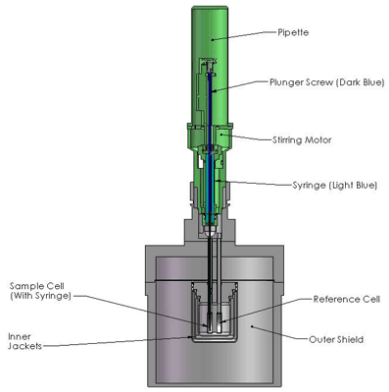
Fig 2(above) and Fig 2a (below).(www.microcal.com).

How does ITC work?

Here we will discuss the working of Microcal's ITC instrument. Microcal's ITC systems use a cell feedback network (CFB) to differentially measure and compensate for heat produced or absorbed between the sample and the reference cell. Twin coin- shaped cells are mounted in a cylindrical adiabatic environment, and connect to the outside through narrow access tubes(figure3). A thermoelectric device measures the temperature difference between the two cells and a second device measures the temperature difference between the cells and jacket. As chemical reactions occur in the sample cell, heat is generated or absorbed . The temperature difference between the sample and reference cells (ΔT) is kept constant by addition or removal of heat to the sample cell as Appropriate, using CFB system. The integral of the power required to maintain ΔT as constant over time is a measure of total heat resulting from the process being studied .

Schematic Diagram of an ITC cell.





Some Examples of Application of Isothermal Calorimetry.

The application of calorimetry for examining hydration effects:-

Water is an important part of DNA and protein structure and therefore it is necessary to account for salvation effects when examining macromolecular stability and biomolecule-ligand interactions. However, the interaction of water and ions with biological molecules is complicated and the thermodynamic consequences of hydration effects can be difficult to rationalize.

One possibility for assessing the role of water in binding interactions as well as nucleic acid/protein stability is to combine the use of calorimetry with the osmotic stress technique. If the macromolecule undergoes a conformational change where the molecules expand and water must be taken up to cover the additional surface area, then osmotic stress will inhibit the conformation change. Conversely, if there is a net loss of solvent exposed surface area, then water must be removed into the bulk solvent and an increased osmotic pressure will favour the process.ITC is a convenient method for obtaining this type of data since the variation of the binding constant with the osmotic pressure (concentration of the osmolyte) can be evaluated. The effects of altering water activity by the addition of co-solutes on melting of duplex and

triplex DNA have been Studied [19.] In this article changes in the number of bound waters as DNA unfolds were evaluated and the concomitant effect of the free energy of DNA melting was determined. The release/uptake of water and counterions has also been addressed using osmotic stress method for Drug-DNA interactions [20,21.].Also TATA binding protein –DNA interactions were studied in which ITC was used to differentiate the effects of water release and cation binding.[22]

ITC Studies on binding of DNA base PNA base to Gold nanoparticles[23.]

In this interesting article, ITC experiment is done on the reaction between DNA and PNA with gold nano particles. It was found that the PNA Bind more strongly to gold nano particles than its corresponding DNA . Also the order of binding for both the DNA and PNA is in order of cytosine> guanine>adenine>thyamine. The binding of Thyamine is very weak compared to the other bases.

It is successfully argued based on literature that Amine groups are known to bind strongly with Aqueous Gold nano particles . Thyamine does not have an exocyclic amine group when compared with others.

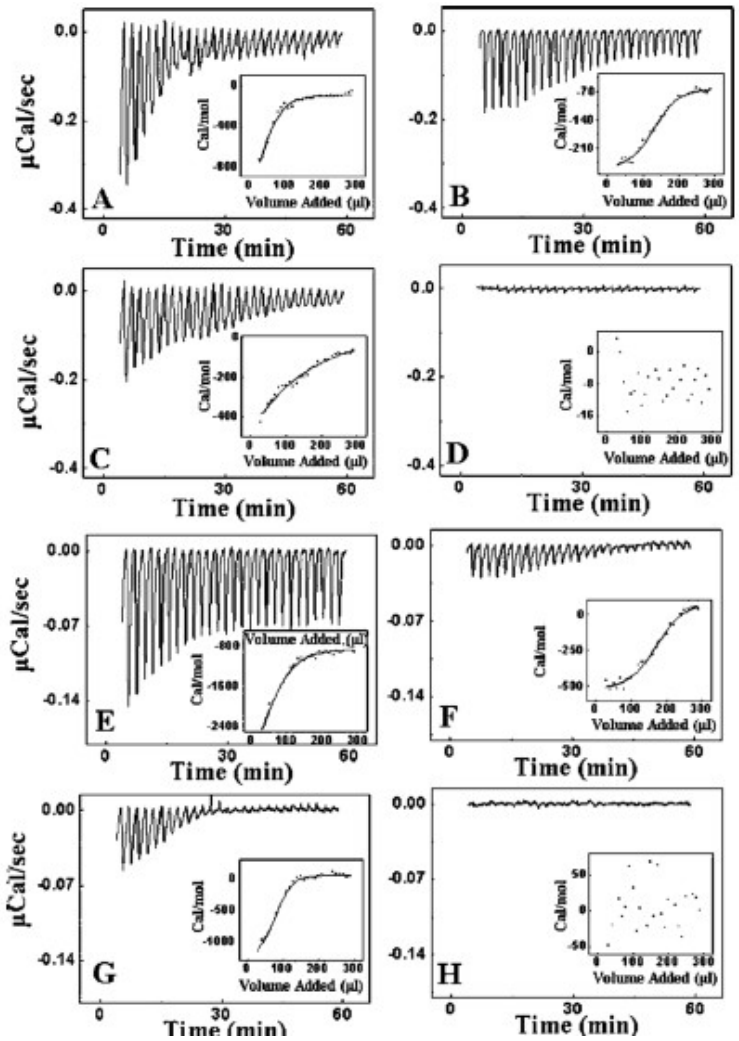


Fig:- ITC curves of binding of DNA and PNA with gold nano particles. D and H represents the scattered pattern of thyamine binding.

ITC studies on ferrous binding to recombinant Human H-chain ferritin(protein)[24.]

Iron deposition within the iron storage protein ferritin involves a complex series of events comprising of ferrous ion binding, transport and oxidation at ferroxidase sites and mineralization of a hydrous ferric oxide core, the storage form of iron.

This study is focussed on the thermodynamic properties of ferrous ion binding to recombinant human H-chain apoferritin(HuHF) using ITC in order to find out about the binding sites.

Calorimetric titrations are used to show that principal locus for Ferrous binding is the ferroxidase centre. It was also found out that more number of iron ions bind at higher pH. Any mutation of

ferroxidase centre eliminates the possibility of binding of ferrous ion. However with wildtype HuHf the ΔH=7.82KJ/mole and K=1.48*E5 which shows a very strong binding and a fairly exothermic character.

ITC Studies on binding of Aminoglycoside Complexation with a DNA –RNA hybrid Duplex[25].

In this study, ITC and other spectrometric techniques were employed to characterize and compare the binding of aminoglycoside paromomycin to three octamer nucleic duplexes :-D1.R2, D1.D2,R1.R2(D represents DNA and R represents RNA).

D1-d(GCCACTGC)

D2-d(GCAGTGGC)

R1-r(GCCACUGC)

R2-r(GCAGUGGC)

Where A.G are purines and U,T,C are pyrimidines.

Based on these studies, these conclusions were found out:-

1.Paromomycin binding enhances the thermal stabilities of the RNA.RNA and DNA.RNA complexes , the magnitude definitely greater than the enhancement of thermal stability of DNA.DNA complexes.

2.Paromomycin binding to all the three octamers occur with intake of protons which depend on the pH .

3.The Affinity of paromomycin for the three host duplexes follows the hierarchy ,RNA-RNA>DNA.RNA>>DNA.DNA>

4. The observed affinity of paromomycin for RNA.RNA and DNA.DNA duplexes decreases with increase of pH.

ITC studies on Thermodynamics of the binding of the C-terminal Repeat Domain of Streptococcus sobrinus Glucosyltransferase-1 to Dextran.[26.]

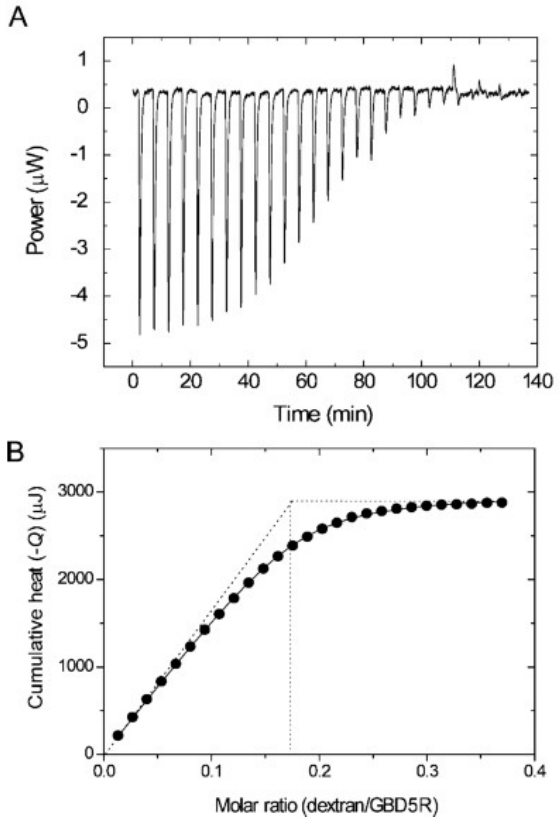
Glucosyltransferases, secreted by mutans streptococci and some other lactic acid bacteria catalyze glucan synthesis from sucrose , and possess a C-terminal glucan binding domain containing homologous , directly repeating units.

In this experiment, series of artificial C-terminal truncated forms of the GBD of *streptococcus sobrinus GTF-1* were prepared and their binding to dextran chain.

It was found our that both the number of glucose units constituting the dextran chain and the enthalpy change associated are proportional to the molecular mass of the truncate.

Also,For the full length GBD(508 amino acid residues),n=33.8,K=4.88E7, and ΔH(std)=-289KJ/mol at 298 K.

The results suggest that identical, independent glucose-binding subsites, each comprising 14 amino acid residues on average ,are arranged consecutively from the GBD N-terminus.



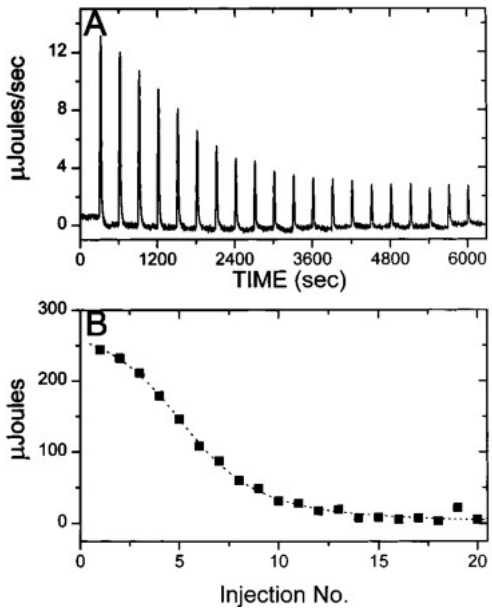
A represents the calorimetric titration of a GBD truncate with dextran.

B represents the Cumulative heat(Q) versus the molar ratio of GBD and dextran. The intersection of the initial slope of the increase in the cumulative heat and the line representing the maximum heat gives the inflection point.

ITC studies on Heparin interaction with a basic cyclic Peptide27.

BNP(Brain natriuretic peptide) is identified as a heparin binding protein on the basis of its cyclic structure and high frequency of basic amino acids, lysine and arginine. ITC experiments demonstrated the transfer of 5 protons from buffer to BNP on heparin binding, suggesting that H-bonding between the polar residues of BNP and heparin is a major factor Contributing to free

energy change of binding. Heparin binding gives a Cp of 1Kcal/mole which suggests burial of polar residues on binding.



Representative binding of heparin to BNP. The fitted data(below) gives a ΔH=-28.7Kcal/mol. Kd=1.78μM and a stoichiometry of 9.92mol of BNP per mole of heparin.

Effect of Temperature on Heparin binding.(for calculation of Cp)

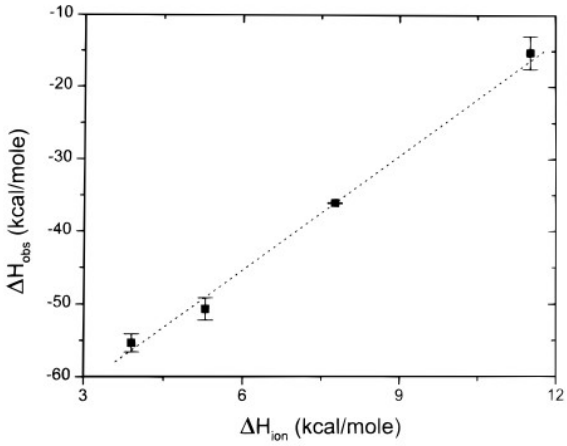


FIGURE 3: Effect of temperature on the BNP−heparin interaction. Linear regression analysis (dotted line) yields a slope (ΔC_p) of 1 kcal/mol and a y-intercept of −361 kcal/mol.

As informed above this curve gives Cp value of 1Kcal/mole which is a very high positive value indicating the burial of polar surfaces.

Effect of salt on heparin binding:-

A polyelectrolyte such as heparin in solution is associated with counterions(+) e.g. Na+ which are released when the binding occurs . The equilibrium relation due to this factor is related as:-

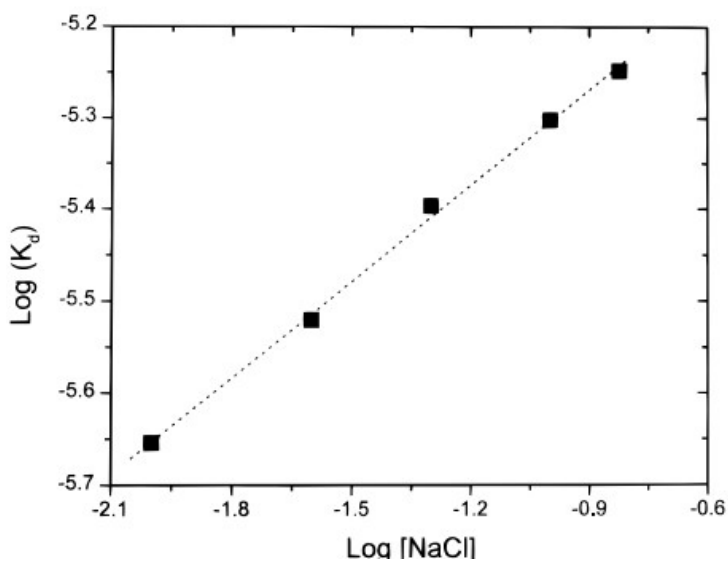
$$\mathrm{BNP}^{N+} + \mathrm{heparin}\cdot N\mathrm{Na}^{+} = \mathrm{BNP}\cdot\mathrm{heparin} + N\psi\mathrm{Na}^{+}$$

Where N is the number of counterions released , and the term ψ represents the fraction of counterion bound per unit charge. Mathematically this has been determined to be0.8 for heparin.

Further It can be shown that the binding decreases as the concentration of salt increases. The relation between Kd and the concentration of salt is given as :

$$\log K_{\mathrm{d}} = \log K_{\mathrm{d(nonionic)}} + N\psi \log[\mathrm{Na}^{+}]$$

The curve plotted for different concentrations and different Kd calculated comes up to be a straight line with a y- intercept of -5 and a slope of 0.4.



Conclusion:-

From different examples we can see that ITC is a powerful instrument for single handed calculation of different thermodynamic parameters.

Technical advances are likely to increase the viability of the ITC technique in more and more research laboratories. The real strength of ITC lies in the fact that it helps in the decision making process in pharmaceutical industries. In choosing from two similar compounds with similar affinities , it has been suggested that the molecule with most favourable enthalpic contribution should proceed for further modifications.

The reason being the association of enthalpy change to energy associated with the net change in non-covalent bonds. Since in development of a drug it is imperative that there should be favourable bonding between the biomolecule and Drug so initial screening of molecules using ITC makes the work easier, cheaper and less cumbersome.

Limitations:-

The only important limitation of ITC machine is that we need to use very high concentrations of Proteins/other substrate for the experiment[28] .

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